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Effect of Plant Growth Promoting Bacteria Inoculation, Addition of Biochar, and Mineral N Fertilization on Mycorrhizal Colonization

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***Abstract*—**Strong anthropogenic impact has uncontrolled consequences on the nature of the soil. Hence, up-to-date sustainable methods of soil state improvement are essential. Investigators provide the evidence that biochar can positively effects physical, chemical, and biological soil properties and the abundance of mycorrhizal fungi which are in the focus of this study. The main aim of the present investigation is to demonstrate the effect of two types of plant growth promoting bacteria (PGPB) inoculums along with the beech wood biochar and mineral N additives on mycorrhizal colonization. Experiment has been set up in laboratory conditions with containers filled with arable soil from the protection zone of the main water source "Brezova nad Svitavou". *Lactuca sativa* (lettuce) has been selected as a model plant. Based on the obtained data, it can be concluded that mycorrhizal colonization increased as the result of combined influence of biochar and PGPB inoculums amendment. In addition, correlation analyses showed that the numbers of main groups of cultivated bacteria were dependent on the degree of mycorrhizal colonization.

***Keywords*—**Arbuscular mycorrhiza, biochar, PGPB inoculum, soil microorganisms.

I. Introduction

ONE of the major ecological threats in the Middle Europe region is the degradation of soil resources influenced by climate conditions and global warming, soil characteristics, topography, changes in land-use and agriculture characteristics [1]. Biochar is a carbon-enriched material made by pyrolysis and intended to be used as a soil amendment due to carbon sequestration and as soil quality enhancer. The C removal from the atmosphere and its safe storage in the soil depths is considered to be a key to avoid sudden and dangerous climate changes. Biochar believes to be a unique opportunity to improve soil fertility and efficiency of nutrients applying locally available and renewable materials in a sustainable way [2].

Mycorrhizal associations produced by *Glomeromycota* fungi are known as arbuscular mycorrhiza, or vesicular-arbuscular mycorrhiza-endotrophic mycorrhiza. Arbuscular mycorrhizas (AM) consider being the oldest and most widespread symbiosis with different plant species [3]. While researchers [4], [5] argue on that AM fungi are multi-functional, it still remains unclear what factors determine which function an AM fungus performs or its respective importance to the plant. Scientists [6] introduce biochar particles as a microhabitat for AM fungi that enables them to survive providing protection from predator grazing as well. In addition, results of the research assumed that biochar might enhance the AM fungi ability to help plants resist fungal pathogen infection. Mycorrhizae are common root-fungal mutualisms with key roles in terrestrial ecosystems and are ubiquitous components in virtually all biomes. Consequently, it is very important to understand how any soil additives, including biochar, may affect their performance [7], [8]. Investigations [9] bring mycorrhizae to light as a sensitive compound to management interventions, such as adding biochar. It is allowed to assume possible synergistic effects of mycorrhizal inoculation and biochar application in enhancing soil quality and plant growth. AM fungi colonize biggest part of the important crop species like maize, rice, wheat, etc. Hence, it may be stated that they are also of a great interest from a perspective of agro-ecosystem productivity and sustainability. The majority of the studies reported in the literature state on applying biochar to soil as a stimulation factor of the crops AM fungi colonization with a strongly positive effect of biochar on mycorrhiza abundance [10]. References [6], [11] stated that AM fungal root colonization has increased in the presence of ground biochar as opposed to non-ground material, and that this effect attributed mainly to the porous structure of biochar. Thus, surface phenomena of biochar and micropore habitats could play an important role in improving mycorrhizal interactions with plant roots; but still all the mechanisms involved are not yet understood. It has been found by [12] that so-called mycorrhization helper bacteria may aid mycorrhizal fungi in colonizing roots. Therefore, this group of facilitative organisms may become stimulated.

It is worth of consideration to investigate deeply how via affecting mycorrhiza biochar could also modify the competitive balance within plant community, biological and biochemical pathways, influence on soil aggregation and consequently the storage of C among these aggregates [13].

The key objective of research is to investigate the biochar amendment influence on arbuscular mycorrhiza (AM) and the inoculums adding effect on roots microscopic fungi- which microorganism communities have development or suppression within these particular biological interactions.

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II. Material and Methods

A. Experimental Design

Experimental research has been provided in laboratory conditions in growth box phytotron with the following conditions: 24°C daily temperature, 20°C night temperature, 65% humidity with a day length of 12 h and light intensity of 380 $\mu mol \cdot m^{-1} \cdot s^{-1}$ in the period from December 2015 until April 2015 (see Fig. 1). *Lactuca sativa* has been chosen as an experimental plant that considers being good indicator of soil conditions changes. Twenty plastic experimental containers have been filled with 1 kg of topsoil from the main protection zone of underground drinking water source "Brezova nad Svitavou". Soil samples have been taken according to ČSN ISO 10 381-6 (ČSN is "Czech Technical Standard"). These experimental samples have been homogenized and have been sieved through a sieve with a grid size of 10 mm. Beech wood biochar have applied into experimental containers with soil. This particular type of biochar is characterized by deriving slow pyrolysis with the use of low temperatures (350°C to 500°C). Five treatments of experiment have been made up to demonstrate biochar, inoculums, and N fertilizers amendment effect with four repetitions in each treatment (see Table I).



Fig. 1 Experimental set up in a growth chamber phytotron

TABLE I
Experimental Treatments of the Applied Treatments

Treatments	Amendment	Application rate	Active ingredients
T1	Control without any additives	-	-
T2	Biochar + "Bactofil" inoculum	50 t ha^{-1} 1 l · ha^{-1}	*Azospirillum brasilense, Azotobacter vinelandii, Bacillus megaterium, Bacillus polymyxa, Pseudomonas fluorescens, Streptomyces albus*
T3	Biochar + "Bactofil" inoculums + DAM 390	50 t ha^{-1} 1 l · ha^{-1} 140 kg N ha^{-1}	*Azospirillum brasilense, Azotobacter vinelandii, Bacillus megaterium, Bacillus polymyxa, Pseudomonas fluorescens, Streptomyces albus, mineral nitrogen*
T4	Biochar + "NovaFerm" inoculum	50 t ha^{-1} 10 l · ha^{-1}	*Azospirillum* spp., *Azotobacter* spp., *Bacillus megaterium, Bacillus subtilis*
T5	Biochar + "NovaFerm" inoculums + DAM 390	50 t ha^{-1} 10 l · ha^{-1} 140 kg N ha^{-1}	*Azospirillum* spp., *Azotobacter* spp., *Bacillus megaterium, Bacillus subtilis, mineral nitrogen*

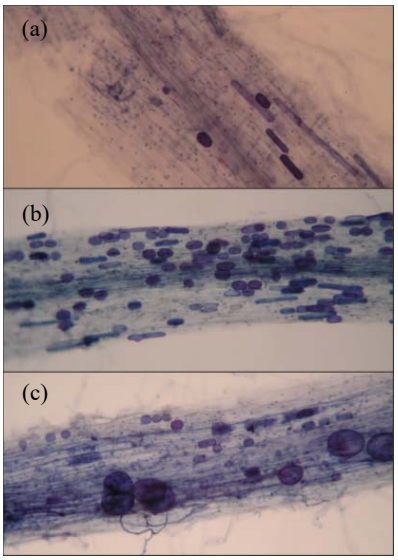


Fig. 2 Stained roots colonization of *Lactuca sativa* by arbuscular mycorrhizal fungi (AMF), (a) Control sample with low AMF colonization, (b) Sample amended with "NovaFerm" inoculum (arbuscules), (c) The same sample colonized by arbuscules, vesicles, extra and intraradical hyphae

It may be seen from the table with the overview treatments that biochar have been added to all the treatments but the first control one. Two types of inoculums have been applied in the four treatments with the difference concerning mineral N in a DAM 390 fertilizer form that contains 30 % of nitrogen; the ratio of ammonium, nitrate, and amidic nitrogen is 1:1:2.

B. Roots Colonization by AM Fungi

Mycorrhizal colonization percentage has been determined in root samples, which have been taken from root system of experimental plant from each container at the end of the experiment. These lettuce roots in 0.5 g of each sample and about 3 cm in length have been washed-out with water and stored in FAA solution (formaldehyde – acetic acid – ethanol 50%). To decrease stain penetration and mycorrhizal roots clearing, samples have been rinsed to remove FAA and incubated with 10% (w/v) KOH solution for 1 h at 90°C. Following is the acidification process with 10 ml of 1 % HCl – sufficient amount to cover root samples in beaker. After this procedure roots are transferred directly into a beaker containing 10 ml of 1% trypan blue in lacto glycerol and are incubated for 1 h at 90°C according to the method [14]. With the next step, stained roots have been cut into 15 mm long segments that have been randomly taken from each plant in 20 repetitions and adjusted to slides. Root segments have been examined by the light microscope Olympus CX-41st.Analyze has been performed with magnification of 100 and for the presence or absence percentage of mycorrhizal fungi arbuscules, vesicles, extra and intraradical hyphae or

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mycorrhizal colonization of roots (see Fig. 2). Percentage of colonization has been calculated as an average of four measurements.

C. Microbiological Analysis

Total number of microorganisms indicative groups including spore-forming bacteria, fungi, actinomycetes and nitrogen-fixing bacteria have been analyzed using colony forming unit method of different bacteria type counting (CFU; dilution plate method). This method has been used for microbial diversity determination in soil samples according to CSN EN ISO 6887-1 (Czech/International Technical Standard – "Part 1 – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination). MPA nonselective medium has been used to estimate total number of microorganisms and spore-forming microorganisms while the latter group has been heated at 85°C for 15 minutes before the seeding on MPA. Czapek Dox agar has been used to determine the number of microscopic fungi, starch and ammonia agar for actinomycetes and Ashby agar for nitrogen-fixing bacteria estimating (Fig.3). Final results have been expressed in CFU per g^{-1}.

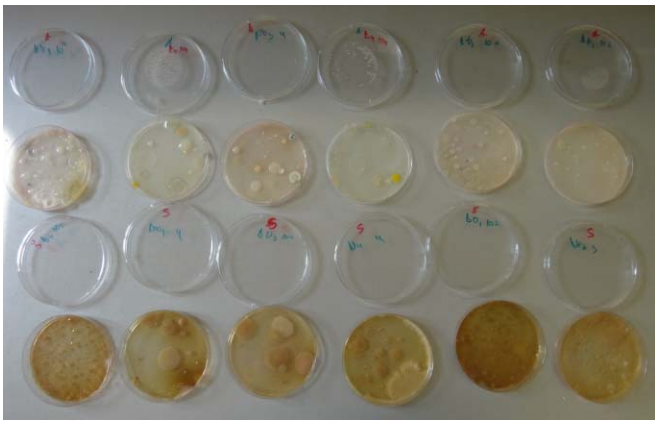


Fig. 3 Microbiological analysis of soil on different nonselective mediums and agars

D. Statistical Analysis

The conceivable differences regarding AM fungi mycorrhizal root colonization have been analyzed by ANOVA analysis with post-hoc Tukey HSD test. All obtained research data have been analyzed in Statistica 12 software. Graphic processing of measured data has been performed in Microsoft Excel 2013.

III. Results and Discussion

A. Assessment of AM Fungi Root Colonization

Several studies demonstrate higher mycorrhizal colonization in the presence of biochar [11], [15]-[18].

Obtained results on AM fungi root colonization demonstrate the trends in Fig. 4.

Data presented in Fig. 4 indicate significant differences in root colonization by AM fungi. Effect of above mentioned soil amendments on AM fungi presence have been noticeable (two-way ANOVA and Tukey test, $p<0.001$). Bright colonization increase have been fixed in the treatments T2 with the application of "Bactofil" inoculum and biochar (48±1%) and T4 – "NovaFerm" inoculum and biochar (52±0.8%) comparing to the control sample (35.6±1.2%). In the contrast treatment T3 with the application of "Bactofil" inoculum, biochar and DAM fertilizer along with treatment T5 with "NovaFerm" inoculum, biochar and DAM fertilizer additives have indicated on a drop of AMF colonization which have resulted in the next values of (33.75±2.3%) and (32.5±1.5%) respectively. It may be assumed that even for the inoculated plants in a combination with nitrogen fertilizers there it is not worth to investigate assimilates through rhizodepozic into symbiotic mycorrhiza as the AM fungi colonization remained at the control level.

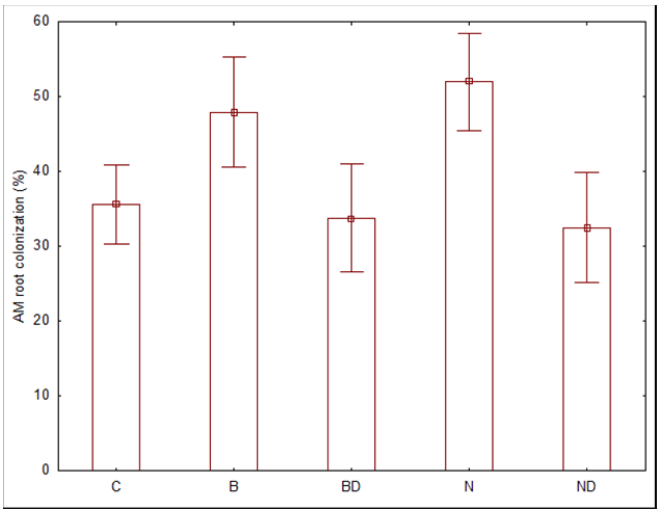


Fig. 4 Root colonization percentage by AMF: Treatment C corresponds to control treatment. Treatments B and BD correspond to treatments with the application of "Bactofil" inoculum and with the same + DAM additive; N and ND are the treatments treated with "NovaFerm" inoculums and the same additive + DAM respectively (mean values ± SD, n = 3)

Reference [19] claims that many AM fungal species vary in their ability to perform each function and exactly these functions can be locally adapted in underground system to limiting soil nutrients. This scientific fact supports our hypothesis regarding inoculums and mineral N fertilizer additives that resulted in higher percentage of AM fungi colonization precisely in the studied treatments without DAM fertilizer amendment.

B. Correlation of Selected Microbial Groups CFU with AM Mycorrhizal Colonization

Reference [20] claimed on the fact that the safe pore environment of biochar might enhance mycorrhizal fungi activity or stimulate so-called mycorrhization helper bacteria.

Table II describes the fluctuated changes in microbial presence of biochar-amended soil, with inoculums additives and mineral N source fertilizer application. Five main groups of microorganisms have been studied: total amount of

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bacteria, micromycetes (microscopic fungi), actinomycetes spore-forming bacteria, and nitrogen-fixing bacteria. Furthermore, correlation analysis between the different ecological groups of bacteria and percentage of AM fungi colonization has been provided (see Table III). Obtained results argued for the high positive correlation coefficients in a case of three groups of microorganisms: actinomycetes up to 0.928, spore-forming bacteria index 0.929 and high correlation with nitrogen-fixing bacteria in 0.906. In contrary, correlation index of bacteria total amount has been average (0.514) and even lower is an index related to fungi (0.105). Reference [2] also apply inoculation with arbuscular mycorrhizal (AM) fungi as a treatment as they state on the fact that in AM pre-inoculated plants disease indices have strongly reduced and even further declined when biochar has been added.

TABLE II
CFU QUANTITATIVE CHANGES IN THE INVESTIGATED SOIL (X FROM EACH VARIANT; N = 3; ± σ)

Treatments	Colony forming units (CFU)				
	Bacteria CFU (106) g-1 soil	Fungi CFU (104) g-1 soil	Actinomycetes CFU (105) g-1 soil	Spore-forming bacteria CFU (104) g-1 soil	Nitrogen-fixing bacteria CFU (104) g-1 soil
T1	5±1.1	5,5±0.3	17±1.6	12±0.5	68.5±4
T2	12±0.6	4±0.2	20±0.6	59±8	140±10
T3	17±0.8	4,6±0.3	13.5±1	18±1.2	53.5±1.2
T4	19.5±3.3	6,25±0.7	29±1	131±3	195±6
T5	9.5±1.5	5,5±0.2	13±0.5	10.5±1.7	101±3

TABLE III
SOIL MICROORGANISMS CORRELATION COEFFICIENTS WITH AM MYCORRHIZA

Groups of microorganisms				
Bacteria CFU (10^6) g-1 soil	Fungi CFU (10^4) g-1 soil	Actinomycetes CFU (10^5) g-1 soil	Spore-forming bacteria CFU (10^4) g-1 soil	Nitrogen-fixing bacteria CFU (10^4) g-1 soil
0.514798	0.105249	0.928966	0.929356	0.906506

IV. CONCLUSION

Reviewing investigation results it may be summarized that inoculated with different groups of microorganisms biochar-amended soil have had definitely positive influence on root colonization by arbuscular mycorrhizal fungi. These suppositions have been proved by the increased values of AM fungi colonization percentage in the studied treatments with two types of inoculums application. In addition, it may be concluded with inoculums positive effect on the main soil ecological groups of microbiota that have resulted in high correlation coefficients within these groups of microorganisms and the percentage of colonization with AM fungi.

Future studies are aimed to investigate biological parameters concerning microorganisms and AM fungi in the same biochar amended soil but with already changed physical and chemical properties due to planting and harvesting the second generation of *Lactuca sativa.*

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